American Journal of Hematology 57:277–282 (1998)

Aspirin Increases the Bleeding Side Effects in Essential Thrombocythemia Independent of the Cyclooxygenase Pathway: Role of the Lipoxygenase Pathway

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Acetylsalicylic acid (ASA) is currently recommended as an antithrombotic for patients with essential thrombocythemia (ET) who are at an increased risk of thrombotic events. However, ASA is also associated with an increased risk of bleeding in these patients as compared to the risk of bleeding in other patients treated with ASA. Recent data suggest that while ASA inhibits platelet thromboxane A_2 (TxA_2) synthesis in all individuals, ASA has little effect or inhibits the lipoxygenase pathway (i.e., 12-hydroxyeicosatetranoic acid or 12-HETE synthesis) in some individuals, and enhances 12-HETE synthesis in others. These differential effects are associated with a pronounced prolongation of the bleeding time vs. no prolongation of the bleeding time, respectively, i.e., in ASA responders and ASA nonresponders, respectively.

To determine if the increased risk of ASA-induced bleeding seen in ET patients is associated with an effect on 12-HETE synthesis, we compared the relative effects of ASA on the bleeding time, platelet TxA_2 and 12-HETE synthesis, and platelet aggregation and adhesion in ET patients and healthy volunteers.

ASA (300 mg, taken orally) prolonged the bleeding time in 82% of the ET patients but only 27% of the healthy volunteers although platelet TxA_2 synthesis and ADP- and collagen-induced aggregation were inhibited significantly in both groups. In contrast, platelet 12-HETE synthesis was unchanged and platelet adhesion was decreased in those patients and volunteers whose bleeding times were prolonged by ASA, whereas platelet 12-HETE synthesis was increased significantly and platelet adhesion was unaffected in those patients and volunteers whose bleeding times were not prolonged, and in some cases shortened by ASA.

These results confirm previous data that demonstrate that ASA has different effects on platelet 12-HETE synthesis and platelet adhesion in different individuals, i.e., inhibitory or no effect in ASA responders (in whom ASA prolonged bleeding) vs. enhancing effects in ASA nonresponders (in whom ASA did not prolong bleeding). These results also indicate that there is a greater percentage of ASA responders in patients with ET than that seen in the general population, a difference that is associated with an effect of ASA on the lipoxygenase pathway. This may explain the increased bleeding side effects seen in the ET patient population. Am. J. Hematol. 57:277–282, 1998.

Key words: essential thrombocythemia; aspirin; lipoxygenase pathway; bleeding side effects

INTRODUCTION

Essential thrombocythemia (ET) is a clonal myeloproliferative disorder that is characterized by an absolute thrombocytosis, which, in turn, is associated with a high incidence of thromboembolic events [1,2]. These events occur predominately in the arterial vasculature, and have

Contract grant sponsor: Heart and Stroke Foundation of Ontario, Canada; Marina Marchetti is the recipient of a fellowship from the Associazione Italiana Ricerca sul Cancro (AIRC).

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Received for publication 29 June 1996; Accepted 12 November 1997

been attributed to quantitative and qualitative platelet anomalies arising from a clonal rearrangement of haematopoietic cells [2]. Several studies suggest that the thromboembolic events occurring in the acral, coronary, and cerebral microcirculation can be prevented by aspirin (acetylsalicylic acid or ASA) treatment, cytoreduction, or a combination of both [3–6]. ASA is given as an antithrombotic on the basis that it inhibits the platelet cyclooxygenase enzyme, thereby preventing the metabolism of arachidonic acid to thromboxane A_2 (TxA_2) [7]. ASA is beneficial for the prevention of thrombotic events because it renders platelets dysfunctional [8], but this ASA effect is associated with an increased risk of bleeding [7]. In patients with myeloproliferative disorders, ASA appears to exacerbate this bleeding side effect more so than in other patient populations [9–11]. This effect has been attributed to a marked effect of ASA on the platelet cyclooxygenase pathway [11].

Recently, it has been demonstrated that ASA has a second effect on the metabolism of arachidonic acid in platelets, which influences platelet function both *ex vivo* and *in vivo* [12]. Thus, ASA inhibits not only TxA_2 synthesis by acetylating cyclooxygenase, but also blocks the synthesis of 12-hydroxyeicosatetraenoic acid (12-HETE) via the lipoxygenase pathway [12]. Inhibition of 12-HETE is associated wtih a decrease in platelet adhesivity independent of the inhibition of the cyclooxygenase pathway [13]. More importantly, ASA only inhibits 12-HETE synthesis and platelet adhesivity in some individuals (ASA responders). These effects are associated with a significant prolongation of the bleeding time. In other individuals, ASA does not inhibit 12-HETE, but rather increases 12-HETE synthesis. In these individuals (ASA nonresponders), platelet adhesion is not inhibited and the bleeding time is unchanged or even shortened [12].

These latter data suggest that the exacerbated bleeding effects of ASA in patients with ET can be attributed to a differential effect of ASA on the platelet lipoxygenase in ET patients as compared to the general population, thereby resulting in a higher ratio of ASA responders: nonresponders in ET patients. To test this possibility, we measured the effects of a single ASA dose (300 mg) on the bleeding time, platelet TxA_2 and 12-HETE synthesis, and platelet aggregation and adhesion in patients with ET and in healthy volunteers. The results demonstrate that ASA, when given in the same dose, prolongs the bleeding time more in more ET patients than in healthy volunteers. These effects are associated with the differential effects of ASA on the inhibition of the cyclo- and lipoxygenase pathways, and subsequent effects on platelet aggregation and adhesion. These results may explain the apparent haemostatic defect seen in ET patients.

PATIENTS AND METHODS

Patients

Seventeen out of 40 ET patients (5 males, 12 females; median age, 49 years, range 26–72), were examined because they were newly diagnosed as having ET according to accepted clinical and laboratory criteria [14]; namely, because they had platelet counts of $\geqslant 600 \times 10^9$ L ($n = 14$), or were off chemotherapy for at least 6 months (3 patients with $<600 \times 10^9$/L platelets). Twenty-three patients were excluded because of chemotherapy (hydroxyurea). None of the ET patients had a bleeding or thrombotic history. The 17 patients were compared to 15 healthy volunteers (8 males, 7 females; median age 51 years, range 30–77). No patient or volunteer had taken any drugs that affect platelet function for at least 3 weeks prior to participating in the study. All participants gave consent after being informed of the objectives, risks, and benefits of participating in the study.

Methods

Blood samples were collected from each individual before and 2 hr after the ingestion of 300 mg ASA (Ascriptin, enteric-coated, Poulenc Rorer, Milan, Italy). The blood was collected into 3.2% trisodium citrate (9:1, v:v). Platelet-rich plasma (PRP) was prepared by differential centrifugation (10 min, 200 g, 22°C). The whole blood and PRP platelet counts were determined by phase contrast microscopy, using the Unopette diluting system, Becton Dickinson, Novate Milanese, Milan, Italy. The platelet count of each PRP was adjusted to 250×10^9 platelets/L, by diluting the PRP with autologous platelet-poor plasma (PPP).

The bleeding time was measured in duplicate at the same time as the blood sample collection, using the automatic Simplate II template device (General Diagnostic, Warner Lambert Co, Morris Plains, NJ). The results of the duplicate measures of each individual (which never differed more than 60 sec) were then averaged. Individuals whose bleeding time was prolonged by ASA >2 standard deviations (SD) from their bleeding time before ASA were defined as ASA responders. Individuals whose bleeding time was not prolonged by ASA, i.e., <2 SD from their pre ASA bleeding time, were defined as ASA nonresponders. The rationale for these definitions are discussed in detail elsewhere [12]. Platelet aggregation was performed at 37°C in an Elvi 840 aggregometer (Elvi Logos, Milan, Italy), using the photometric technique of Born and Cross [15]. Aggregation was induced by adding 15 μM adenosine-5′-diphosphate (ADP, Sigma Chemical Co., St. Louis, MO) or 2 μg/ml collagen (equine fibrillar, Mascia Burnelli, Milan, Italy) to 1 ml

PRP. Aggregation was expressed as maximum % aggregation, measured 3 min after adding the agonist.

The amount of platelet TxA_2 generated in each PRP sample at the end of the aggregation assay was measured as TxB_2, using a radioimmunoassay [13]. The total amount of intraplatelet 12-HETE generated in each sample stimulated with collagen was determined by reverse phase high performance liquid chromatography, as previously described [13].

Platelet adhesivity was measured as the number of ^{3}H-adenine labelled patients adherent to collagen-coated glass coverslips as previously described [16]. Briefly, glass coverslips (18 × 18 mm) were cleaned by ultrasound for 15 min, and left overnight (18 hr) in ethanol. Then the coverslips were rinsed in distilled water. Equine fibrillar collagen (4 μg/cm^2) was sprayed onto each coverslip using an airbrush under a nitrogen pressure of one atmosphere. The coated coverslips were transferred into immulon wells (Falcon, Meylan, France) and incubated with 2 ml of ^{3}H-adenine labelled platelets for 30 min at 37°C. The coverslips were removed from the wells and rinsed three times in HEPES buffer (10 mM in saline) to wash off any nonadherent platelets. The rinsed coverslips with adherent platelets were placed into vials containing 400 μl of Soluene 350 (Packard, Downers Grove, IL) for 18 hr. The amount of ^{3}H was determined by liquid scintillation. The radioactivity was expressed as the number of platelets adherent/coverslip, as calculated from the initial PRP platelet counts and the ^{3}H-adenine platelet specific activity.

Individual platelet suspensions were prepared by separating the platelets from the PRP and washing them twice in Krebs-Ringer solution as previously described [10]. The resuspended platelets were labelled with ^{3}H by incubating them with 50 μCi (10 μM) ^{3}H-adenine (Dupont-NEN, Boston, MA) for 30 min at 37°C. Then the platelets were washed twice in Krebs Ringer solution and resuspended in autologous PPP (pH 7.35) to a platelet count of 250 × 10^9/L. Free adenine (25 μl, 100 μM final concentration) was added to each vial to prevent any nonspecific uptake of ^{3}H-adenine onto the collagen-coated coverslips.

The data were analysed by the Mann Whitney U-test, Chi Square test, and the Wilcoxon test for nonparametric data as appropriate.

RESULTS

The whole blood platelet counts of the ET patients were significantly higher than the platelet counts of the healthy volunteers; 843 ± 287 × 10^9/L and 251 ± 53 × 10^9/L, respectively, mean ± SD, $P < 0.001$. The pre-ASA bleeding times, on the other hand, were not different between the two groups; 5.1 ± 2.7 and 4.9 ± 1.9 min, respectively.

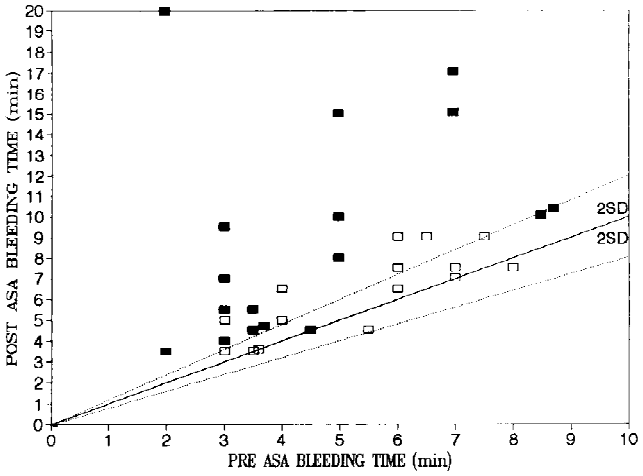

Fig. 1. Prolongation of the Simplate II bleeding time by 300 mg ASA in ET patients (solid squares) and healthy volunteers (open squares). The solid line represents the expected line if there was no change in bleeding times pre and post ASA at any given time point. The dotted lines represent the actual variation (±2 SDs) in repeated measures of the bleeding time when off ASA [12]. Thus, the squares falling above the 2 SD line were considered ASA responders; squares falling within or below the 2 SD lines were considered ASA nonresponders.

When the ET patients ingested a dose of 300 mg ASA, a greater percentage of their bleeding times were prolonged after ASA as compared to the healthy volunteers; i.e., >2 SD or >30% of their pre-ASA bleeding times, $P < 0.001$ and <0.05, respectively (Fig. 1). Moreover, there was a greater prolongation of the bleeding times in the ET patients than in the healthy volunteers. Thus, the bleeding times were prolonged in 82% of the ET patients as compared to only 27% of the healthy volunteers, $P < 0.005$.

Collagen-induced platelet aggregation was inhibited to a similar extent in both groups, $P < 0.001$, irrespective of their bleeding times being prolonged or not prolonged by ASA (Fig. 2, top panel). ADP-induced platelet aggregation was signficantly inhibited in both the ASA responders and nonresponders of the ET patient group, $P < 0.05$. ADP-induced platelet aggregation was not significantly inhibited in the healthy volunteers, although an inhibitory trend was noted. While there were no significant differences in the inhibitory effects of ASA on collagen- and ADP-induced platelet aggregation between patient and volunteer ASA responders vs. nonresponders, inhibition of ADP- and collagen-induced platelet aggregation by ASA was greater in the ET patient group, confidence intervals 0.564–21.556 and 8.449–17.551, respectively.

Platelets obtained from ET patients synthesized more TxA_2 than platelets from healthy volunteers (81 ± 15 vs.

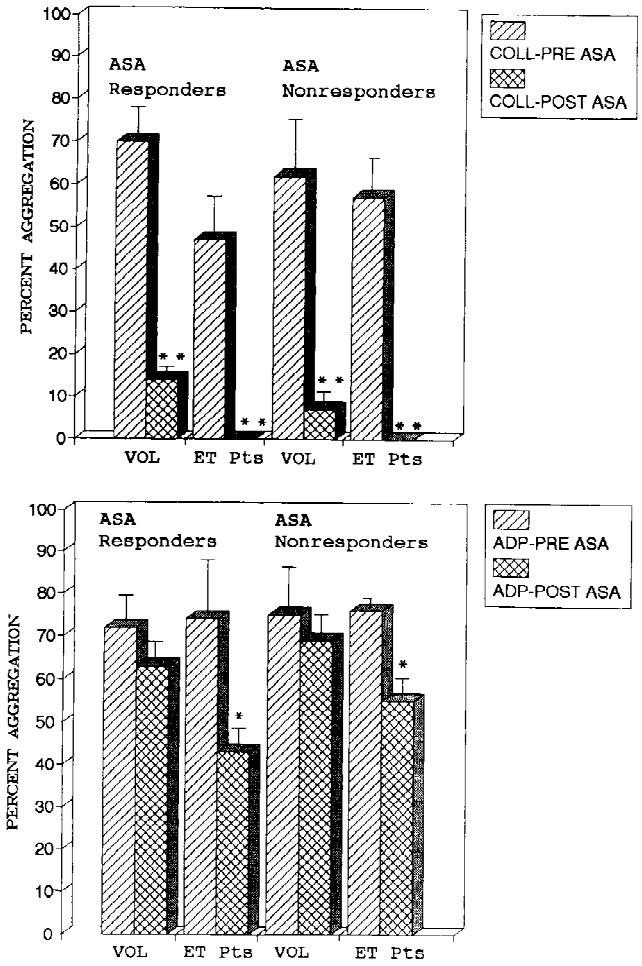

Fig. 2. Inhibition of collagen- (top) and ADP-induced (bottom) platelet aggregation before and after ASA ingestion in ASA responder ET patients (ET Pts) and volunteers (VOL), left sides, and in ASA nonresponder ET Pts and VOL, right sides. Data are expressed as mean ± SD. *$P < 0.05$; **$P < 0.001$.

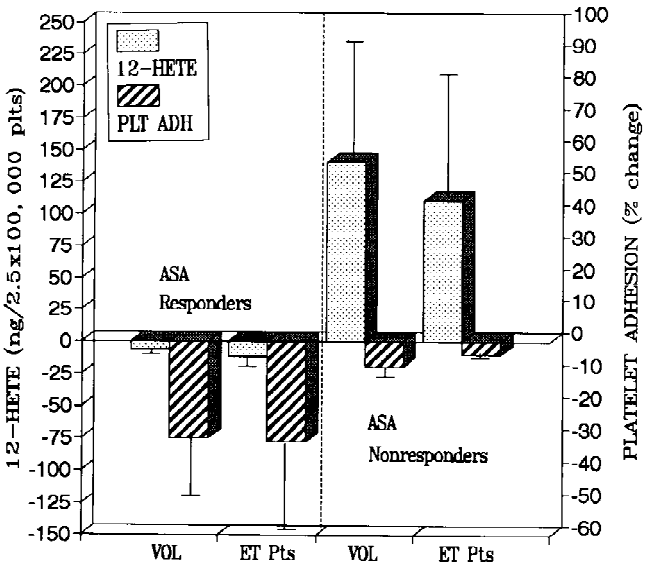

Fig. 3. Effect of ASA on platelet (PLT) adhesion and 12-HETE synthesis in ET patients (Pts) and volunteers (VOL) whose bleeding times (1) were prolonged by ASA (left), and (2) were not prolonged by ASA (right). Data are expressed as mean ± SD.

63 ± 11 ng/10^8 platelets in response to collagen; and 81 ± 23 vs. 18 ± 4 ng/10^8 platelets in response to ADP, $P < 0.05$, respectively). However, ASA inhibited platelet TxA_2 synthesis >95% in all groups, i.e., in ASA responders and nonresponders, regardless of their being ET patients or healthy volunteers (data not shown).

In contrast, there were marked differences in the effects of ASA on platelet adhesion and platelet 12-HETE synthesis in the two groups. Platelet adhesion to collagen was inhibited by ≈30% in those ET patients and volunteers whose bleeding times were significantly prolonged by ASA, $P < 0.01$; i.e., in ASA responders; but platelet adhesion was not inhibited in those patients and volunteers whose bleeding times were not prolonged, i.e., in ASA nonresponders (Fig. 3).

ET patients and volunteers synthesized 17 ± 17 and 24 ± 20 ng of 12-HETE/2.5×10^9 platelets in response to collagen, respectively. There were no differences between the patients or volunteers nor between ASA responders and nonresponders. However, there was little change in intracellular platelet 12-HETE synthesis in the patient and volunteer ASA responders in whom platelet adhesion was decreased, whereas there was a sixfold increase in 12-HETE synthesis in the ASA nonresponders, $P < 0.001$ (Fig. 3); i.e., in those patients and volunteers whose bleeding times and platelet adhesion remained unaffected by ASA despite their platelet aggregations and TxA_2 being inhibited. The lack of a decrease in 12-HETE synthesis and the decreased platelet adhesion in the patient and volunteer ASA responders were significantly different than the increases in 12-HETE synthesis and lack of a platelet adhesion deficit seen in the patient and volunteer ASA nonresponders, $P < 0.001$ and $P < 0.01$, respectively.

DISCUSSION

ASA is used as an antithrombotic agent because its beneficial antithrombotic effect exceeds its side effects [8]. Nonetheless, a certain number of patients are at an increased risk of bleeding, including gastrointestinal haemorrhage [17,18]. Patients with ET who are at risk of thromboembolic events appear to be at an even greater risk of these ASA bleeding side effects when treated with the same ASA dose. This suggests that ET patients respond to the ASA differently than the general population. The results of this present study support the latter possibility and provide a possible explanation.

First, the bleeding time is more prolonged by a fixed

dose of ASA in more ET patients than in healthy volunteers, consistent with the possibility that ET patients are, in fact, at a higher risk of ASA-related bleeding anomalies than the general population [11]. Second, prolongation of the bleeding time is *independent* of the inhibition of TxA_2 synthesis and platelet aggregation, irrespective of individual status (ET patient or healthy volunteer). However, those individuals whose bleeding times are prolonged by ASA (ASA responders [12]) can be distinguished from individuals whose bleeding times are not prolonged by ASA (ASA nonresponders) based upon the differential effects of ASA on platelet 12-HETE synthesis and platelet adhesivity, consistent with previous studies [12]. ASA not only inhibits TxA_2 synthesis and platelet aggregation in ASA responders, it either has little effect or inhibits 12-HETE synthesis, thereby resulting in the inhibition of platelet adhesion as well as inhibition of platelet aggregation in ASA responders [12]. In contrast, ASA appears to enhance 12-HETE synthesis in ASA nonresponders, presumably as a result of an increased amount of arachidonic acid available to the lipoxygenase pathway [19,20]. As a result, platelet adhesion is not impaired.

These data are also consistent with other studies that suggest that 12-HETE plays an important role in cell adhesion, such as increasing polymorphonuclear leukocyte, platelet, and tumour cell adhesion [13,21–23]. Thus, if a 12-HETE is increased by ASA in some individuals, the increased 12-HETE may offset any platelet dysfunction associated with the inhibition of the platelet cyclooxygenase pathway. Several mechanisms have been postulated to explain this: 12-HETE and other related monohydroxides have been shown to influence the expression of adhesive proteins on cell surfaces either directly [24,25], or indirectly by activating the protein kinase pathway involved in integrin expression [26].

Finally, our data suggest that the reason why some ET patients are at a higher risk of ASA-induced bleeding side effects, is because 12-HETE synthesis in ET patients may be more susceptible to ASA-induced inhibition that 12-HETE synthesis in the general population. This may be due to a unique lipoxygenase anomaly as compared to the general population, to differences in platelet membrane permeability that influence access of the salicylate moiety of ASA to the cytosolic lipoxygenase, or to differences in platelet function associated with platelet size. The latter possibility seems unlikely since platelet function per se, i.e., platelet aggregation and adhesion responses, were similar before and after ASA for both ET patients and healthy volunteers when tested at similar platelet plasma concentrations. Moreover, in previous studies we have shown that there is no correlation between the presence of megathrombocytes and platelet responses to a variety of proaggregating stimuli, including ADP and collagen, or with the bleeding time or platelet count in ET patients [27]. It seems more likely that the increased propensity of ET patients to bleed following ASA ingestion is related to their platelets synthesizing less 12-HETE relative to their synthesizing TxA_2 as compared to the healthy volunteers. A number of studies have indicated that patients with myeloproliferative disorders have a lipoxygenase deficit [20,28]. Observations that the platelets obtained from ET patients produce more TxA_2 than the volunteers are also consistent with that possibility [20]. Whatever the explanation, this study suggests that before patients with ET are prescribed the current recommended dose of ASA as an antithrombotic, their increased bleeding risk should be taken into consideration. It is possible that a lower dose of ASA (which may inhibit the cyclooxygenase enzyme effectively [12], e.g., 180 vs. 325 mg) may be warranted. Measuring the bleeding times in these patients before and after ASA may be a useful test to determine this.

ACKNOWLEDGMENTS

These studies were supported, in part, by the Heart and Stroke Foundation of Ontario. We thank Mrs. S. Marziali (Bergamo), Mrs. S. Gataiance, Mrs. L.J. Smith, and Mrs. C. Wale (Hamilton) for their technical assistance. We also thank Dr. G. de Gaetano (Istituto Mario Negri, Consorzio Negri Sud) for his helpful discussions.

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